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Apolipoprotein E isoprotein-specific interactions with tissue plasminogen activator

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Abstract

Apolipoprotein E (Apo E) is an important genetic risk factor for multiple neurological, vascular and cardiovascular diseases. Previously, we reported Apo E isoprotein-specific modulation of tissue plasminogen activator (tPA) using an in vitro blood-clotting assay. Here, we studied the conformational changes of Apo E2, E3 and E4 in the presence of tPA and vice versa using circular dichroism (CD) and dual polarization interferometry (DPI). We report isoprotein and state-specific intermolecular interactions between the Apo E isoforms and tPA. Apo E2 interaction with immobilized tPA leads to significant conformational changes which are not observed with Apo E3 or E4. Additionally, tPA induces changes in helicity of lipidated Apo E2 whereas no detectible changes were observed in Apo E3 or E4. The Tukey's test for interaction indicated a significant ($P < 0.001$) interaction between tPA and Apo E2 in the lipidated environment. These results may be important regarding the mechanism by which Apo E has isoprotein-specific effects on many biological processes and diseases involving blood clotting, proteolysis and perfusion.

Keywords: Apolipoprotein E; Tissue plasminogen activator; Thrombolysis; Protein–protein interaction; Mechanism; Dual polarization interferometry

1. Introduction

Apolipoprotein E (Apo E), a member of a family of lipid-associated proteins, has three common isoforms—E2, E3, and E4, with E3 being the most common isoprotein found in humans [1–3]. These monomeric isoforms combine to form six Apo E phenotypes: E2/2, E2/3, E2/4 E3/3 E4/3 and E4/4 [4]. Apo E2, E3, and E4 each differ by substitutions at residues 112 and 158 with amino acids cys and arg. Isoform specific implications for several neurological, vascular and cardiovascular diseases have been reported [1–3,5]. This has raised questions as to the function or biological activity of isoproteins of Apo E [6–8].

Apo E has been linked to outcome and survival following acute injury of the central nervous system as well as the cardiovascular system [9–16]. The presence of an E4 allele has been associated with a poor outcome following severe head trauma or subarachnoid hemorrhage (SAH) [11,12,15,17] and with reduced survival rates in patients with an intracerebral hemorrhage (ICH) [9]. Others have recently found that there is increased efficacy of tissue plasminogen activator (tPA) in stroke patients that have an Apo E2 phenotype, but no such benefit for an Apo E2 phenotype was demonstrated in placebo-treated patients, as measured either by clinical outcome or by CT-lesion volume at 3 months post-stroke [4]. The putative modification of tPA's activity during acute ischemic stroke

Abbreviations: Apo E, apolipoprotein E; tPA, recombinant tissue plasminogen activator; DPI, dual polarization interferometry; CD, circular dichroism; ICH, intracerebral hemorrhage; TLC, thin layer chromatography

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may be due to Apo E's unique binding properties with factor(s) of the clotting cascade. This change in tPA activity might thus alter the brain's response to reperfusion. This may also provide an important clue for the apparent increased activity of tPA therapy in stroke patients that carry an Apo E2 allele.

Apo E has been found to interact with certain molecules of the clotting cascade. Heparin has been shown to bind to Apo E and decrease Apo E induced neurotoxicity in culture [18–20]. Endothelial production of heparin sulfate has been found to be stimulated by Apo E in vitro [21]. Interestingly, heparin has been suggested to enhance the lytic effect of tPA in stroke patients [22]. We have previously reported that Apo E2 can increase, and Apo E4 can decrease, tPA induced clot lysis in vitro [23]. In those studies, we presented thin layer chromatography (TLC) data that suggested a direct interaction between the tPA and the Apo E isoforms that were distinct between E2, E3 and E4. The isoprotein-specific effects of Apo E (especially that of Apo E2 versus E4) seem to support the suggestion that Apo E isoproteins may have an effect on the blood clot dynamics (formation/degradation) and that this may be due to the direct interaction of Apo E with certain factors in the blood.

Using circular dichroism (CD) and dual polarization interferometry (DPI), the present study examines the direct interactions between Apo E and tPA as a part of the biochemical mechanism through which Apo E may mediate its action on tPA-induced clot lysis. We report an isoprotein-specific interaction of Apo E2 with tPA which is distinct from a relative lack of specific interaction with Apo E3 or E4 in the presence of model membranes, as determined by the CD data. Those findings are corroborated by DPI where comparison with the tPA-E3 data suggests that Apo E2 binds to tPA in a specific manner forming a compacted quaternary structure of the two molecules. Apo E4 binds to tPA in a specific fashion to form a more open complex with tPA than E2. Both of these interactions are distinct from the nonspecific nature of the E3–tPA interaction.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA) without further purification unless otherwise noted. Recombinant tPA (Alteplase) was reconstituted as suggested by the manufacturer, Genentech (San Francisco, CA) and used without further purification. Apo E2, E3 and E4 were obtained and used without further purification from PanVera (Madison, WI).

2.2. DPI

The measurement method employed utilizes two optical waveguides stacked one on top of the other. The waveguides confine light between defined boundaries (cf optical fibers). This vertical structure is shown in Fig. 1. Polarized light from a laser is fed to the end of the stack and therefore at the moment of entry, the light waves in the top and bottom waveguide must be in step (in ''phase''). Upon exposure of the top surface of the upper (sensing) waveguide to a protein that adheres to it, the speed of the light within it is changed. The light in the lower waveguide experiences no such influence and therefore progresses at a constant velocity and provides an optical 'reference'. Thus, when the light exits from each of the waveguides, they are no longer in phase. By simply allowing the output light to diverge from the two waveguides and combine with each other at some distance (in the ''far-field''), a series of light and dark bands (interference fringes) is observed. The precise position of the light and dark bands depends upon the phase relationship of the light as it emerges from the two waveguides. As further protein is added or removed from the sensor surface,

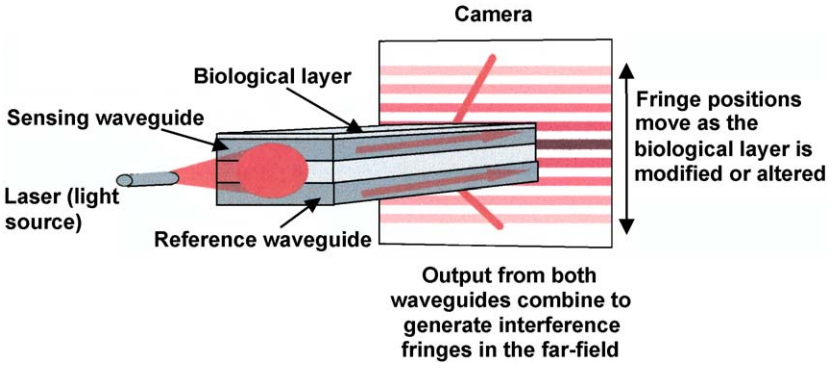

Fig. 1. Schematic of dual polarization interferometer. Two simultaneous optical measurements of the protein are made in orthogonal polarizations of light as they travel *along* the glass slide (rather than perpendicular to it as in a microscope) and the nearfield associated with both polarizations interrogates the immobilized protein. Any changes on the surface of the glass will result in two independent signals from the two polarizations which can be resolved into the size and density of the immobilized protein layer. (For color see online version).

the speed of light through the upper waveguide is further changed and hence the position of the interference fringes move. The phase changes are deduced from the distance of fringe movement.

A second measurement is provided by introducing a second polarization of light, at right angles to the first. This responds differently to protein adsorption/desorption providing an independent second measurement. Using classical optical theory, it is possible to interpret the two measurements in terms of thickness and density for the adsorbed protein. Using this technique, measurements in protein thickness can be made to sub-atomic resolutions in real time with full analysis of the technique published elsewhere [24]. It is important to recognize that this measurement embodies a quantitative analytical technique rather than a simple 'sensor' response function, providing absolute measurements that can be related directly to the structure and function of biomaterials immobilized to the measurement surface.

Analysis of molecular interactions between bound tPA and Apo E was undertaken using an *Ana*Light® Bio200 (Farfield Sensors Ltd, Manchester, UK) dual polarization interferometer. tPA (0.1 mg/ml) in phosphate buffered saline (PBS), pH 7.4, was immobilized to a siloxynitride surface functionalized with amine groups via the amine to amine linker Bis (sulfosuccinimidyl) suberate, 2 mg/ml (BS^3, Pierce, Cheshire, UK). Following establishment of a stable baseline with perfusion of PBS, Apo E isoforms (9.8 μg/ml) were perfused over the immobilized tPA for 3 min, then incubated for 40 min before elution to stable signal. All measurements were performed at 20 °C and captured at a rate of 1 point/s.

2.3. CD

All CD measurements were made on a Jasco J715 at room temperature. Typically, spectra were obtained from 250 to 180 nm with 0.1-nm step resolution at 50 nm/min. The resultant spectra were the average of 4 scans using a band width of 1.0 nm and a sensitivity of 20 mdeg. Each spectrum had the solvent (vehicle) spectrum run separately and subtracted out. For samples where tPA was titrated in, the spectra of the same tPA titration into the vehicle were recorded and subtracted from their respective Apo E spectra. Thus, the resultant spectra are truly a representation of changes in Apo E secondary structure, with no contribution from the spectra of tPA or vehicle. Standard noise reduction of the spectra was performed. Sodium dodecyl sulfate (SDS) micelles were used in 100-fold excess (0.14 mM) of the Apo E as a model membrane system since this more closely simulates the in vivo conditions where Apo E is free to exchange between the lipidated and non-lipidated states. SDS micelles are often used as a model lipid membrane system for structural and conformational studies of proteins and peptides and do not denature proteins under these conditions [25–27].

Typically, commercially available Apo E was diluted further in the supplied media (0.7 M ammonium bicarbonate) to the appropriate protein concentration (1.4 μM) for spectra accumulation, either in the presence or absence of SDS micelles. tPA was then titrated into the cuvette, with spectra collection at each concentration. Concentrations of tPA used were 0, 4.8, 7.1 and 11.7 μg/ml. Changes in ellipticity at 208 and 222 nm were recorded and plotted as a function of tPA concentration as an indicator of changes in secondary structure.

2.4. Statistical analysis

Statistical analysis using the Tukey's single-degree of freedom test for interaction [28] indicated that there is a very significant interaction ($P < 0.001$) between the tPA and the isoprotein in a model lipid environment.

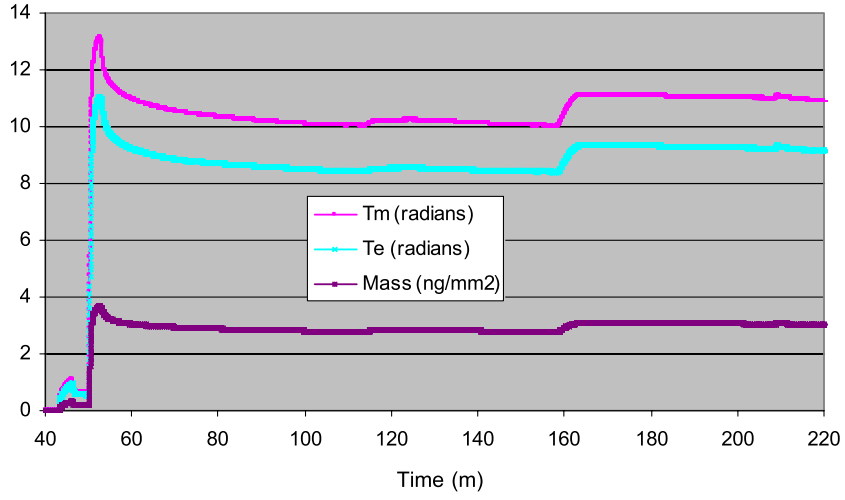

Fig. 2. Representative phase data for tPA immobilization and challenging with Apo E. Phase data for both polarizations, magnetic (Tm) and electronic (Te), and mass analysis of the immobilization of BS^3 (42 min) tPA (50 m) and the subsequent interaction of Apo E2 (160 m). (For color see online version).

3. Results

3.1. DPI

Fig. 2 shows a complete experiment in terms of phase response for both polarizations and, additionally, mass is displayed for a series of deposited layers upon the surface of the sensor component. The tPA immobilization was repeated five times and the average thickness of the immobilized tPA was 4.91 ± 0.55 nm with a surface loading of 2.87 ± 0.09 ng/mm^2 and a density of 0.829 ± 0.096 g/cm^3. Mass and thickness changes occurring when the immobilized tPA surface is challenged with Apo E2, E3 and Apo E4 are shown in Fig. 3. While the mass of bound Apo E is similar regardless of the isoform used, the final layer thickness observed is considerably greater in the case of Apo E3 than in the case of Apo E2 or E4, indicative of Apo E3 showing no specific affinity for tPA, in sharp contrast to the complex formed between tPA and Apo E2. Finally, the density changes which occur when Apo E interacts with the immobilized tPA are shown in Fig. 4. It can be seen that the layer is substantially denser after the addition of Apo E2 while after the addition of Apo E3 or E4, the layer is more diffuse.

3.2. CD

The CD spectrum of Apo E4 in an aqueous solution is shown in Fig. 5 with increasing concentrations of tPA titrated into the solution. Consistent with our previous TLC results [23], in an aqueous (non-lipidated) solution, Apo E4 has an interaction with tPA. Similar interactions were observed when tPA was titrated into an aqueous solutions of Apo E2 or E3 (data not shown). Correspondingly, tPA was titrated into solutions of the Apo E isoproteins in the presence of SDS micelles (lipidated), in order to more closely represent the in vivo conditions where Apo E is free to partition between the free and lipidated states. As a measure of secondary structural changes [29], in Fig. 6 we plotted the ratio of ellipticity at 222/208 nm vs. tPA concentration for each isoprotein. In this model of the in vivo environment, Apo E3 and E4 do not appear to interact with tPA, as observed by the lack of significant secondary structural changes of the apolipoproteins. However, Apo E2 does appear to interact with tPA in a dose-dependent fashion, under these—relatively physiological—conditions. The significance of interaction was mainly due to the 11.7 μg/ml tPA dose. However, even at the 7.1 μg/ml tPA level, the difference was significant ($P < 0.05$). As a control, each of the isoforms was compared in the presence and absence of SDS micelles, and no significant change in conformation was detected (data not shown).

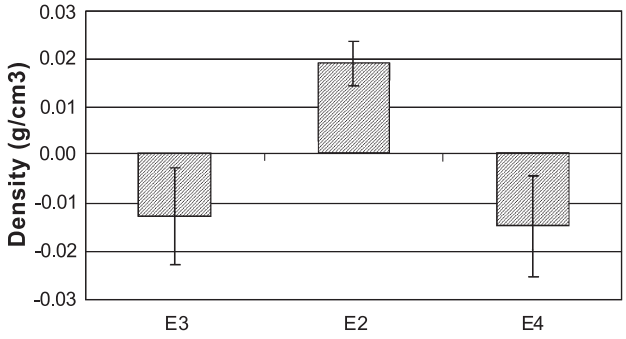

Fig. 4. Density of tPA layer in the presence of Apo E isoforms. The change in density of the immobilized tPA layer upon addition of APO E isoforms, with bars representing range of changes observed. As shown, the protein layer density is increased considerably after the addition of Apo E2 whereas density was decreased with addition of Apo E3 or E4.

4. Discussion

We found that Apo E2, E3 and E4 have distinct isoprotein-specific interactions with tPA. Using two different analytical techniques, we have confirmed our preliminary TLC results [23]. A novel technique, DPI, was used to probe the protein–protein interactions, and found support for those data obtained using the more traditional CD. Below we discuss the impact of these observations and the clinical significance of the isoprotein-specific modulation of tPA activity via Apo E. We make this argument because tPA is

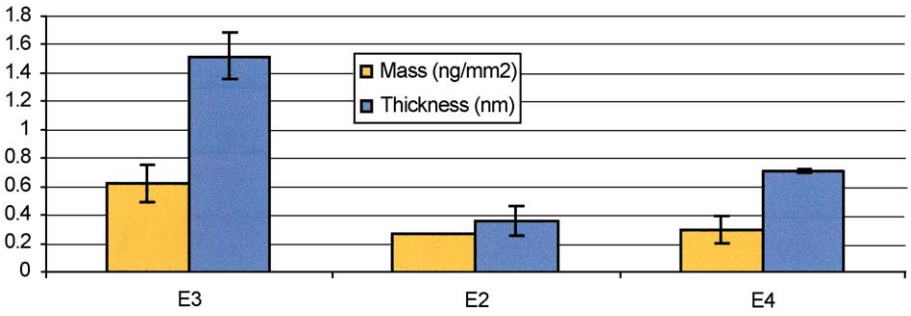

Fig. 3. Mass/thickness of tPA layer in the presence of Apo E isoforms. Mass and thickness changes occurring when immobilized tPA is challenged with Apo E. Bars representing range of data observed, with the bar for E4 mass within the top bar of the data. While the mass of Apo E bound is similar, the thickness of the protein layer is substantially greater for Apo E3 than either E2 or E4. (For color see online version).

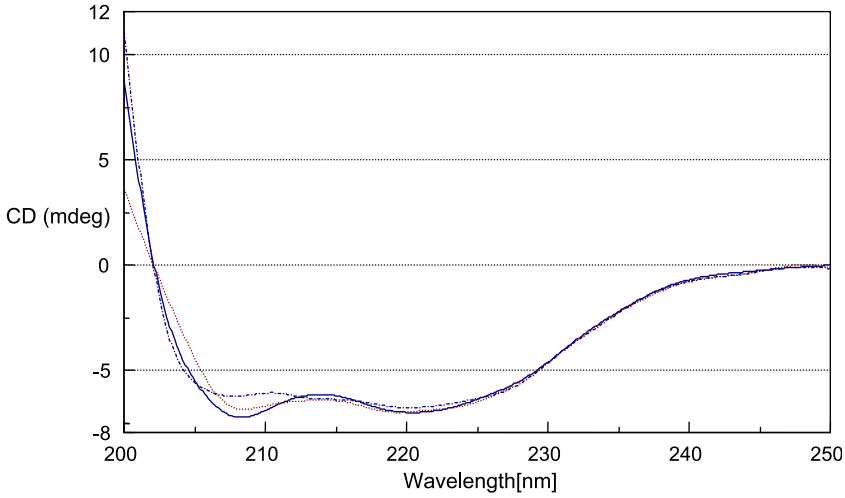

Fig. 5. Representative CD spectra. Here we see the titration of tPA into 0.05 μg/μl Apo E4 in 0.7 M ammonium bicarbonate (in the absence of SDS micelles). tPA concentrations are 0, 0.005 and 0.012 μg/μl for the thin blue, red dotted and thick blue traces, respectively. As can be seen, the typical alpha-helical region of the protein spectra is dose-dependently altered, consistent with intermolecular interactions between the proteins. (For color see online version).

an enzyme and we are showing specific interactions causing conformational changes with the tPA. We infer from these data that the isoprotein-specific interactions between tPA and Apo E are the mechanism through which Apo E modulates tPA's proteolytic activity, resulting in altered thrombolytic and proteolytic degradation.

4.1. Clot lysis

Clot lysis by administration of tPA to stroke patients within 3 h following stroke onset is a beneficial therapy approved by the FDA [22,30–33]. tPA therapy appears to be enhanced with the Apo E2 phenotype [4]. In vitro evidence indicates that Apo E4 is associated with a decrease in the lytic action of tPA and that Apo E2 is associated with an increase in lytic action [23]. It is likely that these results are due to reduced or enhanced breakdown of fibrin caused by Apo E modulation of tPA induced proteolysis, respectively. tPA administration shifts this process (of clot formation and clot degradation) to favor clot degradation and Apo E2 enhances the apparent activity of tPA, while Apo E4 inhibits the tPA induced proteolysis.

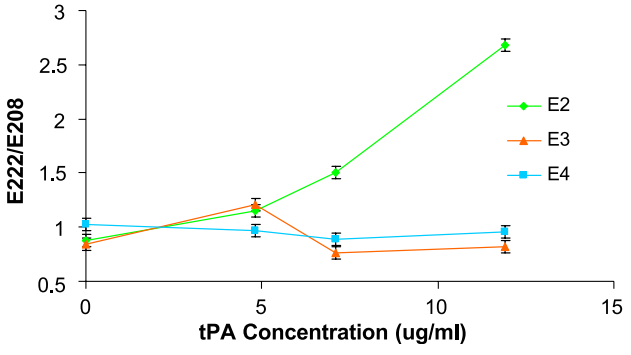

Fig. 6. Molar ellipticity ratio as a function of tPA concentration. Ratio of molar ellipticity at 222 to 208 nm as a function of tPA concentration for Apolipoproteins E2, E3 and E4 in the presence of SDS micelles. An indicator of 2° structure within the protein, this ratio suggests that tPA interacts specifically with Apo E2, altering E2's secondary structure under these model membrane conditions. However, tPA does not appear to alter the structure of Apo E3 or E4 under the same conditions, indicating that the interactions are likely due to nonspecific, hydrophobic interactions, consistent with the DPI results (Figs. 3 and 4), or that the interactions simply do not elicit a conformational changes in the apolipoprotein. (For color see online version).

Specifically, in our previous study, tPA-induced clot lysis (stimulated by exogenous tPA) was significantly enhanced by supplementation with Apo E2 (EC_{50} of 0.20 ± 0.06 μg/ml) as compared to tPA alone (0.72 ± 0.19). However, when Apo E4 was supplemented to the clot lysis assay, there was a significant inhibition of clot lysis (EC_{50} of 0.98 ± 0.23 μg/ml) whereas there was no significant change in tPA induced clot lysis caused by Apo E3 [23]. Thus, the three isoproteins of Apo E had distinct effects on thrombolysis in vitro. This phenomenon, while interesting, did not determine whether there was a direct and isoprotein-specific interaction between Apo E and tPA. However, our findings could be explained by direct interaction of Apo E and tPA resulting in the isoprotein-specific changes in tPA activity.

We also found that Apo E2 added to freshly obtained blood, in the *absence* of exogenous tPA, reduced the overall clot formation in vitro with no effect attributed to E3 or E4. As tPA is an endogenous protease involved in the clotting cascade, we believe our results are indicative of the exogenous Apo E2 enhancement of the endogenous tPA induced clot lysis. Additionally, TLC experiments suggested an interaction between Apo E2 and E4 with tPA in an aqueous environment that was distinctly different than that of Apo E3 with tPA [23]. In the current report, we found that the isoproteins may have different propensities for interacting with tPA in a lipid environment (Fig. 6). These results are consistent with other work showing that the apolipoprotein

isoforms have distinct affinities for lipoproteins, receptors and lipids [34,35].

In this study, we set out to determine the mechanism by which Apo E isoproteins might have their effects on tPA induced clot lysis. Within the body, there is a dynamic partitioning of the apolipoproteins between the free and lipidated state. This partitioning is also likely to be occurring in the presence of the SDS micelles, thus making this model of lipidated versus non-lipidated proteins well suited for studying these protein–protein interactions. For these studies, DPI and CD were used as appropriate and sensitive analytical methods for probing the differences in the protein–protein interactions between tPA and Apo E.

4.2. DPI

DPI is an emerging analytical technique which is capable of precise measurements of the thickness and density of material attached to the surface of an optical sensor chip. The technique has been used to measure protein immobilization [24a], self-assembly of molecules (such as surfactants [24b]) and ligand binding to proteins. It has also been used to measure specific binding events which typically involve an increase in density and decrease in thickness (typically caused by conformational changes) as binding takes place.

The data from the two polarizations in Fig. 2 are used to calculate the thickness and density of material on the chip surface. The data from the two polarizations are combined to generate the data in Figs. 3 and 4. When the change in the immobilized protein layer upon addition of the protein isoforms is compared, it is clear that despite similar masses of Apo E being bound to the tPA, the thickness and density of the final layers are notably different. In the case of Apo E2, the layer is considerably thinner and denser than is the case for Apo E3 or E4. This suggests that the final structures of the three are significantly different with tPA and that there is a more intimate, and specific, interaction between tPA and Apo E2 compared to the other two isoforms. The complex formed between tPA and E4 is more diffuse than that of E2–tPA, but the thickness and density values suggest a different interaction from that of E3–tPA, with E4 binding to tPA in a specific fashion to form a more open complex with tPA than E2. These interactions are clearly distinct from the nonspecific nature of the E3–tPA interaction.

4.3. CD

The use of CD for detecting conformational changes in proteins has been well established [36,37]. The CD spectrum of proteins reflects the types and amounts of secondary structure within the protein. For example, helical structures have double minima at 208 and 222 nm, whereas β-sheet structures elicit a single minimum at 216 nm. In contrast, random coil conformations yield a minimum at 200 nm. When these areas of secondary structure are altered, such as when a ligand binds, the CD spectrum is also altered. The alterations in CD spectra can be used to determine which specific regions of a structure undergo a conformational change. In this way, the CD data can be used to map the areas of interaction between two proteins. As has been described and used by others [37,38], we used the ellipticity ratio $\Theta_{222}/\Theta_{208}$ to measure the differences in interaction between Apo E2, E3 and E4 with tPA.

From Fig. 5, we see that in an aqueous environment, Apo E interacts with tPA, as judged by dose-dependent conformational changes in the secondary structure of the protein. This result is not surprising, however, as proteins in an aqueous environment are likely to interact due to various reasons, including nonspecific hydrophobic interactions [39]. Fig. 6 plots the ratio of the ellipticity at 222:208 nm vs. tPA concentration for each of the Apo E's in a lipid environment. This type of plot can be used as an indicator of conformational changes, typical of intermolecular interactions [37,38]. The implications for Fig. 6 can be one or a combination of two things: that Apo E2 has a higher propensity for interacting with tPA in a lipid environment than E3 or E4, or that Apo E2 has a lower propensity for incorporation into the SDS micelles, or some combination of both. In either case, the isoproteins appear to interact distinctly with tPA and in an isoprotein and state-dependent manor.

4.4. Isoprotein effects of Apo E

There is a growing body of evidence that Apo E isoproteins have numerous clinically relevant actions [40–43]. There are many isoprotein-specific conditions, including: cerebral amyloid angiopathy (CAA), Alzheimer's disease (AD), heart disease, atherosclerosis, ICH and poor outcome following head trauma and stroke. Arguably, all of these conditions relate, in some way, to alterations in the normal functioning of the clotting cascade and/or blood flow. Taken together with our results, it appears as though apolipoproteins E2 and E4 may play an active role in the mechanism of disease and vascular dysfunction by altering tPA induced proteolysis.

Many of the diseases found to be genetically or correlatively linked to the Apo E isoproteins are generally considered to be conditions of chronically compromised blood flow and, hence, older age. It is possible, in the case of Apo E4, that chronic inhibition or reduction of endogenous tPA potency may mediate the deposition of proteins such as amyloid β-peptide, as in CAA and AD. Indeed, it was recently found that the tPA-plasminogen cascade is involved in the normal clearance of Aβ [44]. It was therein stated that "reduced activity of this system may contribute to the progression of Alzheimer's disease" [44]. Further, reduced clot breakdown can lead to ICH via the resulting increase in pressure in the microvasculature. Conversely, in the case of Apo E2, where tPA potency is increased, continual high rates of flow and protease activity can lead

to thinning of the blood vessels and susceptibility to damage from shear stress, contributing to ICH and heart disease.

The apparent interaction of Apo E with tPA can affect clot degradation, and/or tPA's proteolytic activity in several ways. First, it could bind to the tPA, directly affecting its clot degradation activity by increasing or decreasing its ability to cleave plasminogen to plasmin. In the same way, the activity of tPA has been shown previously to be altered by binding to vascular smooth muscle cells with concomitant changes in conformation [46]. Alternatively, the clot lysis activity could be altered by enhancing or inhibiting the inactivation of tPA by plasminogen activator inhibitor 1 (PAI-1). This is important because PAI-1 is an established modulator of tPA activity via direct and specific protein–protein interactions [45]. Our isoprotein-specific interactions of Apo E with tPA compliment the studies by Aleshkrov et al. [45] who showed that fragments of PAI-1 had distinctly different binding capacities for tPA. To that end, we have preliminary results (data not shown) indicating that the effects on clot lysis of the Apo E's are specific to tPA. The activity of the closely related thrombolytic Tenecteplase (Genentech), a derivative of tPA engineered specifically to be more stable to PAI-1 inhibition, does not appear to be significantly affected by the presence of any of the Apo E's. These preliminary results, along with those shown here, appear to indicate that Apo E mediates it effects on clot lysis through isoprotein-specific, direct interactions with tPA. More work is needed to determine the precise mechanistic effects Apo E has on tPA's conformation/kinetic activity.

5. Conclusions

From these studies, we conclude that Apo E interacts directly with tPA in an isoprotein and state-specific manner, potentially explaining the difference in their effects on tPA-induced clot lysis. Explicitly, we found that Apo E2 has a specific, intimate interaction with tPA in lipid and aqueous solutions as judged by the CD and DPI data, whereas the interaction of Apo E4 with tPA specific, but does not form a compacted overall structure as E2 does. Furthermore, Apo E3 does not appear to have a specific affinity for interacting with tPA other than potentially through hydrophobic interactions. The interactions of Apo E2 and E4 could directly affect the ability of tPA (either endogenous or exogenous) to cleave plasminogen to plasmin, therefore altering thrombolysis and proteolysis dynamics.

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